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**RGD-FasL 的基因构建、表达、纯化
及功能分析**

**Construction, Expression and Purification of RGD-FasL and
Analysis its Function**

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摘要

恶性肿瘤是严重威胁人类生命健康的头号杀手,手术、放疗和化疗是治疗肿瘤的常用方法。放疗、化疗因同时杀伤肿瘤细胞和正常细胞,副作用大,因此寻找特异、高效的肿瘤治疗方法一直是肿瘤研究的热点。肝癌是危害人类健康的常见恶性肿瘤,肝癌是所有肿瘤中的第二位致死因素,肝癌的治疗方法及其对肝癌防治至关重要,近年来,随着肝癌的发病机理和治疗研究的进展,人们认识到细胞凋亡机制的异常在肝癌发生和抗肿瘤治疗中起重要作用,研究发现很多抗肿瘤治疗方法是通过诱导肿瘤细胞凋亡发挥作用,因此,寻找能够诱导肝癌细胞凋亡的活性物质,通过激发肝癌细胞的凋亡途径,达到治疗肝癌的目的是肝癌防治的有效方法之一。

由于 FasL 具有抗肿瘤作用和多种免疫调节功能,FasL 疗法的临床研究已在许多国家开展。动物实验和临床实验均表明,FasL 对某些肿瘤具有明显的抑制作用;但是由于副作用较大,为 FasL 的临床应用造成困难。患者常因不能耐受其毒副作用而终止用药。为了降低 FasL 的副作用,临床多采用局部用药、联合用药、温热疗法等。尽可能降低 FasL 用药量,增强疗效而不损伤正常细胞,然而,欲从根本上解决 FasL 的疗效问题还需要对 FasL 进行遗传改造以获得高特异性、低毒性的新一代 FasL,有关这方面的工作,目前主要集中在以下两个方面:一是根据 FasL 结构与功能的关系研究结果,通过遗传操作,获得 FasL 突变体,降低其毒副作用;二是将 FasL 与其它蛋白共同构建双功能的融合蛋白,实现肿瘤靶向治疗。^[2]肿瘤靶向治疗是利用特异性的载体或导向系统,将药物或其他杀伤肿瘤细胞的活性物质选择性地运送到肿瘤部位,而不影响正常细胞、组织或器官的功能,从而提高疗效、减少毒副作用。肿瘤靶向治疗中治疗靶点、靶向载体和效应分子的选择是关键。

整合素是一类膜受体家族,由 α 和 β 两个亚单位组成。在部分肿瘤细胞或者肿瘤新生血管内皮细胞中,常特异性地高表达某些整合素,如 $\alpha v \beta 3$,而正常细胞不表达或表达量很低。整合素 $\alpha v \beta 3$ 在肿瘤新生血管形成、肿瘤生长和转移过程中发挥重要作用,可以作为一类新的肿瘤治疗靶点;RGD 肽是一类含有精氨酸-甘氨酸-天门冬氨酸(Arg-Gly-Asp)的短肽,细胞外基质(ECM)和血液中的粘附蛋白是人体中最常见的含 RGD 序列的蛋白。研究发现,大部分整合素与其配体

的结合过程,是通过识别配体中所含的 RGD 序列来完成的。目前,利用噬菌体表面展示技术,已经筛选出与一类与整合素 $\alpha v\beta 3$ 特异性结合的 RGD 肽,即 ACDCRGDCFCG(RGD-4C),这类 RGD 肽可以作为肿瘤靶向载体,特异性地引导抗肿瘤药物到达肿瘤部位,杀灭肿瘤细胞。

本研究以整合素 $\alpha v\beta 3$ 为靶点, RGD 肽为靶向载体, FasL 蛋白为抗肿瘤效应分子,构建双靶向性抗肿瘤融合蛋白 RGD-FasL,并检测其生物学活性,为深入探讨 RGD-FasL 的体内靶向抑瘤活性及肿瘤治疗应用前景奠定基础。同时丰富了 FasL 在肿瘤治疗中的应用形式,为肿瘤靶向治疗提供了新思路。

我们获得 RGD-FasL 基因,构建到表达载体 pGEX-5X-1,通过酶切、测序鉴定阳性重组载体。含重组质粒 pGEX-5X-1/RGD-FasL 的 *E.coli* BL21DE(3) 经 IPTG 诱导表达后,通过不同的诱导时间和诱导剂浓度对重组菌进行诱导表达,确定其最适表达条件为 IPTG 终浓度为 0.5mmol/L、30℃诱导 12 h。目的蛋白主要存在包涵体裂解液上清中,表达量占菌体总蛋白的 30%以上,过 GST Resin 柱纯化。经 SDS-PAGE 分析在 Mr 62 000 附近有一特异条带,符合目的蛋白理论推算值 (Mr 约 62 700),扫描分析显示其纯度达 95%以上,透析和稀释复性制备正确折叠的融合蛋白。通过流式细胞仪检测肝癌分子表面 Fas,CD61 的表达,通过体外粘附实验证实 RGD-FasL 具有靶向性,从而为进一步实验提供了有力基础。MTT 比色实验证实, RGD-FasL 能抑制小鼠肝癌细胞 H22 和人肝癌 H9101 细胞生长,对正常小鼠肝细胞生长没有影响;通过 Annexin V-FITC/PI 双染流式细胞术定量分析技术和末端脱氧核苷酸转移酶介导的 dUTP 缺口末端标记 (TUNEL) 方法分析证实 RGD-FasL 诱导 H22 和 H9101 细胞凋亡能力;体内实验显示 RGD-FasL 融合蛋白能有效抑制鼠源性肝癌的生长。在 H22 小鼠模型中,当 RGD-FasL 剂量为 2mg /kg 时, RGD-FasL 的抑瘤率为 62.5%,优于相同剂量的 FasL 的抑瘤率 (51.3%);免疫组化及 HE 染色组织学观察发现 RGD-FasL 能选择性作用于肿瘤组织,诱导肿瘤细胞凋亡,而对正常组织及肝组织没有毒副作用。

本研究表明:融合蛋白 RGD-FasL 具有诱导肝癌细胞凋亡,抑制肝癌细胞生长的能力。同时 RGD-FasL 能够特异性结合肿瘤细胞,具有肿瘤靶向性,有利于发挥 FasL 的抗肿瘤活性。为深入探讨肝癌的基因治疗提供了新的方法与途径。

关键词: FasL ; RGD-FasL; 肿瘤靶向性

Abstract

The malignant tumor seriously threaten the health of mankind. The commonly used cancer therapy methods include surgery, radiotherapy and chemotherapy. However, the side effect of radiotherapy and chemotherapy is serious, because normal cells were simultaneously killed. Carcinoma of the liver is the common malignant tumor that does harm to the human beings' health. liver cancer is the second position lethal factor in all tumors, which is next only to lung cancer in city or cancer of the stomach in rural. Therefore the research of treating liver cancer is urgent. Now surgical operation, chemotherapy, radiotherapy and biotherapy are four kinds of main ways to treat it, In recent years, with progress of the pathogenesis and treatment research of liver cancer, it has being known that the unusual mechanism of cell apoptosis occurring in liver cancer cells, which would be of importance to develop new treatment method. Recently, we have been trying to induce cancer cell apoptosis for Anti-cancer therapy. Tumor targeting therapy is a novel method which selectively kills tumor cells by means of specific tumor targeting vehicles, without harming normal cells, and is a central challenge for improving cancer therapies.

FasL is cytotoxic to various tumor cells and involved in immunological regulation. But when used in clinical, its side effects are usually intolerable. So its usage is limited. In recent years, many works have been done to widen FasL's clinical use by ①the construction of FasL mutants; ②the construction of bi-function fusion Proteins with other Proteins; Tumor targeting therapy is a novel method which selectively kills tumor cells by means of specific tumor targeting vehicles, without harming normal cells, and is a central challenge for improving cancer therapies.

Integrins are a family of cell surface receptors with α and β subunits. Integrin $\alpha v \beta 3$ expressed on proliferating but not on quiescent endothelial cells. So integrin $\alpha v \beta 3$ can highly expressed on endothelial cells of tumor blood vessels or tumor cells. It is a new target for cancer therapy. The RGD (arginine-glycine-aspartic acid) sequence is known to serve a recognition moiety between the ligands and integrins. Present data obtained

from phagdisplay techniques found that ACDCRGDCFCG(RGD-4C) can bind selectively to $\alpha\beta 3$ integrin. So RGD-4C is a promising homing peptide for tumor targeting therapy.

In this work, we fused FasL with the RGD peptide, a ligand of $\alpha\beta 3$ integrins, by recombinant DNA technology. We constructed E.coli expression system of the fusion protein RGD-FasL. Then we studied its dual activity in vitro, inducing tumor cells apoptosis (H22 and H9101) and the property of tumor targeting.

We amplified the fusion gene RGD- FasL by PCR, then cloned the gene RGD- FasL into the express vectors pGEX-5x-1. The recombined vectors were identified through restriction endonuclease analysis and gene sequencing. A single positive *E.coli* BL21DE(3) of pGEX-5X-1/RGD-FasL clone was incubated in shaken flasks at 30 °C in LB medium for 12 h, inducing by 0.5mmol/L IPTG. Several conditions, such as IPTG concentration and inducing time, temperature of induction were optimized. The proteins were expressed mainly as inclusion bodies with the yield of more than 30% of total bacterial proteins. The fusion protein was purified by GST affinity column (according to the manufacturer's protocols of Gen Script Corporation) and identified by SDS-page electrophoresis. On SDS-PAGE, the mobility of the purified protein was found to correspond to a molecular weight of 62 kDa as expected and no degradation was observed. After purification, the purity of the proteins were all more than 95%.

MTT demonstrated that the fusion protein RGD- FasL could induce tumor cells (H22 and H9101) apoptosis and suppress the growth of tumor cells in vitro. The expression of Fas and CD61 on the surface of hepatoma cells were assayed by FCM. And we study on adhesive properties of RGD-FasL in vitro. To quantify apoptosis, we utilized the annexin V-FITC staining assay, which reports the loss of phosphatidylserine asymmetry of plasma membrane at the early stage of apoptosis. TUNEL methods were used to detect and quantify myocardial apoptosis in all experimental cell lines, based on labeling of DNA strand breaks. In the H22 (mouse liver cancer)-bearing mouse models, the tumor growth inhibitions of RGD- FasL were 62.5%, which is better than group treated with FasL (growth inhibitions 51.3%). In

histological studies, the selective localization of the fusion proteins was observed. and tumor cells around the occluded vessels appeared damaged. No thrombosis in blood vessels and cell damage, or other side effects were observed in normal tissues.

We can conclude that the fusion protein RGD- FasL could induce tumor cells apoptosis and result in growth suppression in vitro, without harming normal cells, and have the characteristic of tumor targeting. This study shed light on the possible usage of RGD-FasL in further investigation of hepatocellular carcinoma, which provides a voluble way for the design of new gene therapy.

Key words: FasL; RGD-FasL; tumor targeting

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前言

原发性肝癌是一种恶性程度很高的肿瘤,在我国有很高的发病率,据卫生部统计,肝癌年死亡率在城市和乡村中分别占据恶性肿瘤死亡率的第二和第一位。且其发病率一直居高不下,对肝癌的研究已成为肿瘤研究领域的热门课题目前,肝癌的治疗主要采取手术切除为主、化放疗为辅的综合治疗方式。但中晚期肝癌的治疗效果尚不能令人满意,肝癌手术切除后复发率较高。所以,伴随着分子生物学技术的发展,生物治疗已成为继外科,放化疗之后的第四种肝癌治疗模式^[1]鉴于细胞凋亡异常在许多恶性肿瘤的发生学上占有十分重要的地位,我们相信随着对凋亡机制的深入研究,有望在肿瘤的治疗上取得新的突破。以选择性的诱导肿瘤细胞为目标的凋亡干预技术可能成为治疗肝癌的基本策略。

肝癌的发生具有多病因、多阶段性。细胞凋亡障碍在恶性肿瘤的发生、发展中起关键作用,细胞增殖过度,凋亡受到抑制均会导致肿瘤。肝细胞癌变过程中癌基因和相关基因的激活,在肿瘤形成过程中起重要作用。多种参与细胞凋亡的信号通路和调控的基因异常,使细胞无限制生长,导致肿瘤发生。Fas 是细胞表面重要的死亡受体,是细胞凋亡的信号分子。Fas 与其配体 FasL 结合,活化并传导凋亡信号,是诱导细胞凋亡的重要途径。肿瘤的发生和发展与细胞凋亡的异常有密切的关系,其中,通过 Fas/ FasL 系统使肿瘤细胞抵抗 Fas 介导的凋亡以及反击免疫细胞使 T 细胞凋亡,是肿瘤细胞免疫逃避的重要机制之一^[2,3]。深入探讨肝癌细胞凋亡现象及机制,为肝癌的基因治疗提供新的思路与途径。

一、Fas/ FasL 系统及其诱导的凋亡在肝癌发生的重要地位

1 Fas/ FasL 系统及其诱导的凋亡

Fas 和 FasL 相互作用是诱导细胞凋亡的重要途径之一。Fas 也称 Apo21 或 CD95,是属于肿瘤坏死因子受体(TNFR) / 神经生长因子受体(N GFR) 家族的 I 型跨膜糖蛋白。Fas 抗原主要以膜受体形式存在,也可通过在转录水平 mRNA 的不同拼接使翻译产物缺失跨膜区形成可溶性 Fas(sFas),存在于胞浆和血清中。sFas 与配体 FasL 结合,可阻断 FasL 与膜受体型 Fas 结合,从而对细胞凋亡起抑制作用。与相应细胞比较,肿瘤细胞通常 Fas 表达低下或完全不表达,但也有细胞

恶性转化后新出现 Fas 表达。FasL 是 Fas 在人体内的天然配体,是属于 TNF 家族的 II 型跨膜蛋白。^[2,3]FasL 主要表达于激活的 T 细胞、NK 细胞、部分肿瘤细胞以及一些免疫豁免区如眼前房、睾丸滋养细胞表面等。^[4,5]膜结合型 FasL 在金属酶裂解作用下成为可溶性 FasL (sFasL) ,其介导凋亡的能力比膜结合型减弱。Fas 与 FasL 结合可触发凋亡,其凋亡信号的传递途径为: FasL 以三聚体形式与靶细胞上的 Fas 结合,诱导 Fas 三聚化,使 Fas 胞浆区的死亡结构域(DD) 与转接器蛋白的 Fas 相关死亡域(FADD) 结合,形成死亡诱导信号复合体(DISC) ,DISC 的形成可以诱导 FADD N 端的死亡效应区(DED) 与前半胱天冬氨酸蛋白酶 8 (procaspase28) N 端的 DED 相互作用,形成活化的 Caspase28 ,后者再通过直接或间接途径裂解和活化 Caspase 家族的其他分子如 Caspase2 ,1 ,3 ,6 ,7 ,激活 Caspase 级联反应,Caspases 通过对底物降解可转导凋亡信号或直接作为凋亡的效应分子促进细胞骨架降解和 DNA 片段化,导致细胞凋亡的发生。^[6,7]

2 Fas 表达水平与肝癌发生

目前对肝癌组织中 Fas 表达水平的报道结果不一。Hamazaki 等^[8]发现肝癌中 Fas 表达明显高于正常肝脏和慢性肝病组织,而 Strand 等^[9]的实验结果则与之相反。目前多数学者倾向于认为肝癌 Fas 表达降低或缺失。Nagao 等^[10]发现肝癌患者的非癌肝组织细胞 Fas 表达上调,肝癌细胞则下调;与 Fas 阴性病例比较, Fas 阳性的肝癌患者肝内转移灶少,肿瘤细胞凋亡率高,患者存活时间长;肝癌患者血清中 sFas 明显增高。因此认为,肝癌细胞可能通过下调 Fas 表达,并使 sFas 产生增多来逃避机体免疫监视,发生转移。Yano 等^[11]发现,多数肝癌细胞株仅表达胞浆 Fas ,对激活性 Fas 抗体介导的凋亡不敏感;而少数既表达胞浆 Fas 也表达胞膜 Fas 的肝癌细胞株对 Fas 抗体介导的凋亡敏感。提示胞膜 Fas 在细胞凋亡过程中起重要作用。某些肿瘤细胞对 Fas 介导凋亡的敏感性与细胞表面 Fas 的水平并不呈正相关。肿瘤细胞抵抗 Fas 介导凋亡的机制是多方面的,除了 Fas 表达下调或缺失外,还包括肿瘤细胞产生对自身胞浆内 Fas 蛋白表达决定域的截断;凋亡抑制基因的高表达,如 bcl22 家族、Fas 相关的磷酸酶 1 (FAP21) 、FADD 样白细胞介素 β 转化酶(FL ICE) 抑制蛋白(FL IP)以及许多凋亡抑制因子(IAP) 家族成员的上调等等^[12、13]。Natoli 等^[14]研究用的 6 个肝癌细胞株均表达 Fas ,但只有 1 个细胞株对 Fas 抗体介导的凋亡敏感。他们用放线菌酮处理细胞,使对 Fas

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